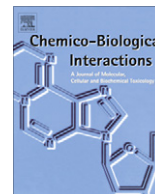


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Modulation of cholinergic pathways and inflammatory mediators in blast-induced traumatic brain injury

Manojkumar Valiyaveetil^{a,*}, Yonas A. Alamneh^a, Stacey-Ann Miller^b, Rasha Hammamieh^b, Peethambar Arun^a, Ying Wang^a, Yanling Wei^a, Samuel Oguntayo^a, Joseph B. Long^a, Madhusoodana P. Nambiar^{a,*}

^aBlast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA

^bUnited States Army Center for Environmental Health Research, United States Army Medical Research and Materiel Command, Fort Detrick, MD 21702, USA

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ABSTRACT

Cholinergic activity has been recognized as a major regulatory component of stress responses after traumatic brain injury (TBI). Centrally acting acetylcholinesterase (AChE) inhibitors are also being considered as potential therapeutic candidates against TBI mediated cognitive impairments. We have evaluated the expression of molecules involved in cholinergic and inflammatory pathways in various regions of brain after repeated blast exposures in mice. Isoflurane anesthetized C57BL/6 J mice were restrained and placed in a prone position transverse to the direction of the shockwaves and exposed to three 20.6 psi blast overpressures with 1–30 min intervals. Brains were collected at the 6 h time point after the last blast exposure and subjected to cDNA microarray and microRNA analysis. cDNA microarray analysis showed significant changes in the expression of cholinergic (muscarinic and nicotinic) and gammaaminobutyric acid and glutamate receptors in the midbrain region along with significant changes in multiple genes involved in inflammatory pathways in various regions of the brain. MicroRNA analysis of cerebellum revealed differential expression of miR-132 and 183, which are linked to cholinergic anti-inflammatory signaling, after blast exposure. Changes in the expression of myeloperoxidase in the cerebellum were confirmed by Western blotting. These results indicate that early pathologic progression of blast TBI involves dysregulation of cholinergic and inflammatory pathways related genes. Acute changes in molecules involved in the modulation of cholinergic and inflammatory pathways after blast TBI can cause long-term central and peripheral pathophysiological changes.

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1. Introduction

Blast exposure has been described as one of the major factors involved in mild to moderate brain injury in service members returning from Iraq and Afghanistan which can lead to chronic neurological disabilities [1–4]. Acute changes in the central and peripheral nervous systems after blast TBI can exacerbate the pathological outcomes resulting in long-term chronic effects [5,6]. Neuroinflammation including cross-talk between central and peripheral immune systems is considered to be a primary event after blast exposure exacerbating the brain injury [6,7]. Inflammation and innate immune responses are primarily regulated by neural mechanisms [8,9]. In particular, cholinergic systems involving the neurotransmitter acetylcholine and the enzyme acetylcholinesterase (AChE) have been proposed as components of an anti-inflammatory pathway regulating neuroimmunomodulation [8–11].

Recently we reported regional specific alterations in the brain AChE activity after repeated blast exposures [12]. AChE inhibitors are possible therapeutic candidates against Alzheimer's disease and TBI [13–15]. In this study, we analyzed the expression of cholinergic and inflammatory related genes in different regions of the brain of repeated blast exposed mice using cDNA microarray. We also analyzed the microRNA expression profile in the cerebellum of blast exposed mice. MicroRNAs are endogenous tissue specific non-coding ribonucleic acids of approximately 18–26 nucleotides which modulate gene expression by binding to complementary mRNA, either targeting degradation or inhibiting translation, potentially play major roles in neuropathophysiology.

2. Materials and methods

2.1. Materials and animals

Trizol reagent, Tris–Bis gradient gels (4–12%), protein molecular weight markers, and SDS–PAGE running and transfer buffers were purchased from Invitrogen Life Technology (Carlsbad, CA); tissue

* Corresponding authors. Tel.: +1 301 319 9679/7307; fax: +1 301 319 9404.

E-mail addresses: m.valiyaveetil@amedd.army.mil (M. Valiyaveetil), madhusoodana.nambiar@amedd.army.mil (M.P. Nambiar).

protein extraction reagent and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL); acetylthiocholine, tetra monoisopropyl pyrophosphotetramide (iso-OMPA), and 4,4'-dipyridyl disulfide, 4,4'-dithiodipyridine (DTP) were purchased from Sigma-Aldrich (St. Louis, MO); polyvinylidene difluoride (PVDF) membrane and anti-myeloperoxidase (MPO) antibody were purchased from Millipore (Billerica, MA). C57BL/6J mice (male, 8–10 weeks old, 21–26 g) were purchased from Jackson Laboratory, Bar Harbor, ME. Animal experiments were performed at Walter Reed Army Institute of Research (WRAIR) in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council Publication, 1996 edition) with an approved Institutional Animal Care and Use Committee protocol. Isoflurane anesthetized mice were exposed to 20.6 psi blast overpressure for three times with 1–30 min intervals as described earlier [5,12,16].

2.2. Brain acetylcholinesterase activity assay

Brain samples collected at various time points (3, 6, 24 h and 3, 7, 14 days) were dissected into different parts (frontal cortex, hind cortex, hippocampus, cerebellum, mid brain and medulla) and homogenized with tissue protein extraction reagent at 4 °C using a tissue homogenizer and centrifuged. AChE activity in the supernatant of brain extracts were measured by using modified Ellman assay with 1 mM of acetylthiocholine substrate and 0.2 mM DTP as chromogen in the presence of 4 µM of iso-OMPA, a butyrylcholinesterase inhibitor, as described earlier [12,17–19]. Brain AChE activity was expressed as milliunits/mg protein.

2.3. Microarray analysis

Various regions of the brain (frontal cortex, cerebellum, mid brain, and hippocampus) of sham and repeated blast exposed mice at 6 h time point after the last blast exposure were collected and total RNA was isolated using Trizol reagent according to manufacturer's protocol. The concentration and purity of RNA were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (5 µg) was labeled using the Agilent Low Input Quick Amp labeling Kit in conjunction with the Agilent two-Color Spike-Mix according to the RNA Spike-In Kit protocol and amplified in a thermal cycler (Mycycler, Bio-Rad Laboratories, Hercules, CA). Labeled RNA samples were subjected to fragmentation followed by 17 h hybridization against universal mouse reference RNA (Stratagene, La Jolla, CA) using the Agilent Gene Expression Hybridization Kit. Agilent 60-mer whole mouse genome 44 K oligo microarrays (Agilent Technologies, Santa Clara, CA) printed with Agilent SurePrint technology were used for microarray analysis as outlined in the Two-Color Microarray-Based Gene Expression Analysis (version 6.5) protocol. Microarray slides were scanned using an Agilent G2565CA fluorescence dual laser scanner for Cy3 and Cy5 excitation and the generated data were feature extracted using default parameters in Agilent Feature Extraction Software (version 10.7.1). GeneSpring 10.1 Software was used to carry out the data filter and normalization.

2.4. Western blot analysis of MPO expression

Total protein was extracted from the cerebellum of sham and repeated blast exposed mice at 6 h time point using tissue protein extraction reagent and the total protein content was estimated by using BCA assay kit. Equal aliquots (30 µg) of protein extracts were separated on 4–12% SDS-PAGE, transferred to PVDF membranes

and probed with anti-MPO antibody. Blots were developed by using chemiluminescent substrate, photographed by Alphamager (CellBioSciences, Santa Clara, CA) and quantified by using ImageJ software.

2.5. MicroRNA analysis

For the microRNA analysis, total RNA was extracted from cerebellar region of sham and repeated blast exposed mice (6 h time point after the last blast exposure) as described above followed by analysis by µParaflo™ MicroRNA microarray at LC Sciences (Houston, TX). Briefly, 4–8 µg total RNA was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, version 16) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. After RNA hybridization, tag conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

2.6. Data and statistical analysis

Statistical analysis of brain AChE enzyme activity was performed by using GraphPad Prism software with Mann-Whitney test. The statistical analysis of the microarray data was performed by using GeneSpring 10.1 Software. Differentially regulated genes (between sham control and blast exposed samples) were selected using Welch's *t*-test analysis ($p < 0.05$), followed by the Benjamini-Hochberg multiple correction test to find genes that varied between control and blast exposed samples with a false discovery rate of 5%. To account for the small sample size, we used the reference design and filtered for genes with signal intensities that are twice the standard deviation of the background intensity levels. We determined that by performing gene-by-gene *t*-tests, for a samples size of 3% and 5% false discovery rate and a standard deviation of 0.5, the power is 75%. We also applied pathway and gene ontology analyses that offer extra power because it is statistically unlikely that a larger fraction of false positive genes end up in one specific pathway.

3. Results

3.1. Summary of AChE activity changes in different regions of brain after blast exposure

Changes in AChE activity in different regions of brains following repeated blast exposure have been reported earlier and the summary is shown in Fig. 1 [12]. Except for frontal cortex, all other brain regions of blast exposed mice showed an acute decrease in the activity of AChE. In the cerebellum and midbrain regions, a significant increase in AChE activity was observed at 3 days, while the medulla region showed a significant increase in AChE activity at

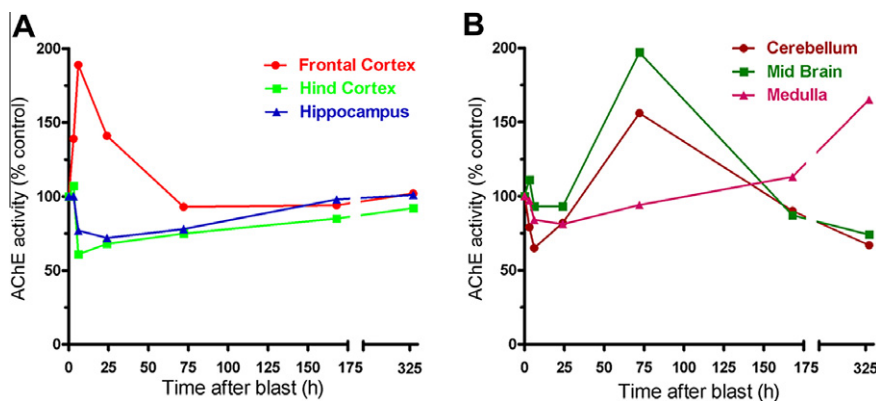


Fig. 1. Summary of AChE activity changes in various regions of the brain after blast exposure. Brain AChE activity of sham and blast exposed mice were analyzed as described in "Section 2". The values were expressed as % of sham control and plotted over time (h). Shown are the mean values from 6 different blast exposed mice.

Table 1

Changes in the expression of cholinergic and inflammatory pathways related genes in various regions of brain after repeated blast exposures.

GenBank accession#	Gene symbol	Gene product	Fold change*	Significance (p value)**
<i>Mid brain</i>				
NM_203491	Chrm2	Cholinergic receptor muscarinic 2	−2.01	0.032
NM_007390	Chrna7	Cholinergic receptor nicotinic alpha 7	−1.53	0.015
NM_008071	Gabrb3	Gammaaminobutyric acid receptor beta 3	−1.38	0.035
NM_008074	Gabrg3	Gammaaminobutyric acid receptor gamma 3	−1.43	0.034
NM_008174	Grm8	Glutamate receptor metabotropic 8	−1.54	0.026
NM_008365	Il18r1	Interleukin 8 receptor 1	−1.42	0.049
NM_009425	Tnfsf10	TNF superfamily 10	−1.65	0.035
NM_021456	Ces1	Carboxylesterase 1	+1.94	0.006
NM_031168	Il6	Interleukin 6	+1.58	0.020
NM_008374	Il9r	Interleukin 9 receptor	+1.48	0.007
NM_139299	Il31ra	Interleukin 31 receptor A	+1.65	0.035
<i>Frontal cortex</i>				
NM_008356	Il13ra2	Interleukin 13 receptor alpha 2	−2.76	0.033
NM_008354	Il12rb2	Interleukin 12 receptor beta 2	−2.47	0.043
NM_021349	Tnfrsf13b	TNF receptor superfamily 13b	−1.36	0.019
NM_134437	Il17rd	Interleukin 17 receptor D	+1.20	0.012
NM_008371	Il7	Interleukin 7	+1.48	0.047
NM_009423	Traf4	TNF receptor associated factor 4	+1.11	0.045
<i>Cerebellum</i>				
NM_177396	Il28	Interleukin 28	−1.54	0.004
NM_010824	Mpo	Myeloperoxidase	+1.56	0.019
NM_011614	Tnfsf12	TNF superfamily 12	+1.95	0.038
NM_178931	Tnfrsf14	TNF receptor superfamily 14	+1.82	0.016
NM_175649	Tnfrsf26	TNF receptor superfamily 26	+1.53	0.036
<i>Hippocampus</i>				
NM_008366	Il2	Interleukin 2	−1.29	0.030
NM_028075	Tnfrsf13c	TNF receptor superfamily 13c	+1.64	0.021

* Average values from 3 different animals ($n = 3$ for sham and blast).

** Significance was calculated using Welch's *t*-test analysis followed by the Benjamini–Hochberg multiple correction test.

14 days post-blast. These data indicate heterogeneous changes in brain AChE activity after blast overpressure.

3.2. Changes in the expression of cholinergic pathway related genes in the midbrain

Gene expression analysis of the midbrain of blast exposed mice using cDNA microarray identified the modulation of multiple genes involved in cholinergic transmission (Table 1). The expression profiles of cholinergic receptors muscarinic 2 and nicotinic alpha 7 were down-regulated after the blast exposure. Similarly, expression of gammaaminobutyric acid receptors beta 3 and gamma 3 was also down-regulated in blast exposed mice. The expression of glutamate receptor metabotropic 8 showed significant reduction after blast exposure.

3.3. Changes in the expression of inflammatory pathway related genes in various brain regions of blast exposed mice

The cDNA microarray analysis showed significant changes in the expression of tumor necrosis factor (TNF), interleukins (IL) and their multiple receptors after blast exposure (Table 1). Several IL receptors showed significant up-regulation in the midbrain. In the frontal cortex, cerebellum and hippocampus the expression of IL receptors was significantly low, while TNF and its receptors showed higher expression (Table 1). There was a significant increase in the expression of carboxylesterase in the midbrain region and myeloperoxidase expression in the cerebellum of blast exposed mice indicating increased inflammation. These results indicate that repeated blast exposure leads to modulation of multiple inflammatory pathway related genes.

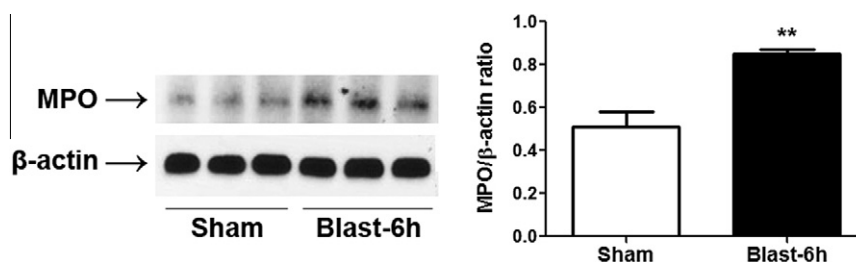


Fig. 2. Western blot analysis of MPO expression in the cerebellum after blast exposure. Cerebellar regions were collected from sham and repeated blast exposed animals at 6 h time point and the total protein was extracted, separated on 4–12% Tris–Bis gels and immunoblotted for MPO expression as described in “Section 2”. Blots were re-probed with β -actin antibody and quantified using ImageJ software. Shown are the mean \pm SEM values of densitometric measurements of Western blot data. $n = 3$ for sham and blast; and (**) $p < 0.01$.

4. Discussion

The cholinergic anti-inflammatory pathway has been proposed as a link in neuroimmunomodulation, especially during stress conditions [8–11]. Neuroinflammation is one of the major causes for increased neuropathology and neurobehavioral changes after single or repeated exposures to blast overpressure [6,7,22–25]. Our recent studies on the brain regional specific alterations of the activity of AChE in repeated blast exposed mice prompted us to evaluate gene expression related to cholinergic and inflammatory pathways [12]. The results presented in this paper describe significant alterations in the expression of multiple receptors involved in cholinergic and inflammatory pathways after blast exposure.

The significance of cholinergic pathways (nicotinic and muscarinic) in controlling both central and peripheral inflammation has been thoroughly reviewed by Pavlov et al. [9–11]. In our experiments, we found significant down-regulation of cholinergic receptors muscarinic 2 and nicotinic alpha 7 in the midbrain of mice exposed to repeated blasts (Table 1). Previous studies indicate that acetylcholine receptors muscarinic and nicotinic alpha 7 are essential elements of the anti-inflammatory pathways [9–11]. Down-regulation of these receptors in the midbrain region after blast can induce the pro-inflammatory pathways leading to neuropathology and neurobehavioral deficits. Concurrently, GABA and glutamate receptors in the midbrain are down-regulated after repeated blast exposure. Additional studies using RT-PCR and Western blotting with specific antibodies need to be performed to assess the levels of target mRNAs and proteins after blast exposure. These results indicate the modulation of cholinergic and other neurotransmitter signaling pathways after blast exposure which can potentially play multiple roles in the neuroinflammatory process.

Pro-inflammatory cytokines and chemokines are reported to be released both centrally and peripherally after blast exposure [7,22,26]. The cDNA microarray analysis of various brain regions of repeated blast exposed mice showed significant up-regulation of multiple ILs, TNF and their receptors confirming these observations (Table 1). The pro-inflammatory marker, myeloperoxidase expression was significantly higher in the cerebellum of blast exposed mice, which was further confirmed by Western blotting (Table 1 and Fig. 2) [6]. More studies are required to understand the differential effects of blast exposure on gene expression in various regions of the brain. Initial studies indicate that frontal cortex and cerebellum are the two brain regions that may be affected more by blast exposure [3,16]. Also, it is possible that acute effects of blast may be different than chronic effects in various regions of the brain. In addition to blast shock wave (primary blast injury) there can be also a component of acceleration mediated injury (tertiary blast injury) [27]. Thus, blast exposure using shock tube may cause differential effects and the brain damage may not be uniform.

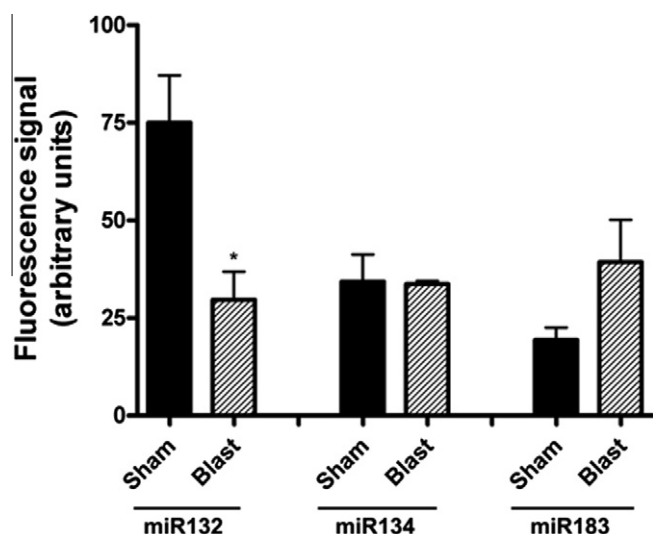


Fig. 3. MicroRNA expression profile in the cerebellum of blast exposed mice. Animals were exposed to 20.6 psi blast overpressure, at 6 h time point the cerebellum was collected by necropsy and the tissue samples were for processed for microRNA analysis as described in “Section 2”. Shown are the mean \pm SEM values of fluorescence intensity expressed in arbitrary units. $n = 3$ for sham and blast; and (*) $0.05 < p < 0.01$.

3.4. MPO expression in the cerebellum after blast exposure

Western blot analysis of MPO in the cerebellum showed significant increase at 6 h after blast exposure (~1.6-fold increase) (Fig. 2), confirming the results from cDNA microarray data (Table 1).

3.5. Changes in microRNA expression in the cerebellum of blast exposed mice

It has been reported earlier that the cerebellum is injured more than other brain regions after blast exposure [5,16]. MicroRNA analysis of blast exposed mice showed significant reduction in the cerebellar expression of miR132, which is reported to be involved in cholinergic anti-inflammatory signaling pathway (Fig. 3) [20]. The expression of miR183 in the cerebellum of blast exposed mice also showed an increase which was not statistically significant. MicroRNA 134 expression showed no significant change after blast exposure. Both miR183 and 134 are involved in stress related cholinergic transmission (Fig. 3) [21]. These results indicate that blast exposure modulates microRNAs involved in both cholinergic and inflammatory pathways after blast exposure.

MicroRNAs have been studied as regulators of cholinergic anti-inflammatory signaling pathways [20,21]. MicroRNA 132 is reported to attenuate the inflammatory process by targeting AChE activity [20]. Significantly decreased expression of miR132 in the cerebellum of blast exposed mice indicates augmentation of pro-inflammatory signaling cascades probably through cholinergic pathways (Fig. 3). There are multiple splice variants of AChE (AChE-S, -R, and -E), among which AChE-R (R stands for 'read-through') is usually generated during stress conditions [28]. Similarly, stress is also reported to up-regulate the expression of splicing factor SC35 which is involved in the formation of AChE-R from AChE-S (S stands for 'synaptic') [21,29]. In our studies, no significant changes in the expression of miR134 and 183 were observed after blast exposure. Both the microRNAs 134 and 183 are known to share a common predicted mRNA target encoding the splicing factor SC35 [21,29]. If there was significant modulation of these microRNAs after blast exposure, it would support the previously reported up-regulation of miR183 and 134 during acute stress targeting two different transcription factors (ZPFM2 and CBFA2T1), which are involved in oxidative stress, inflammation and neuropathology [21,30]. More studies are required to conclude whether neuroimmunomodulatory miRNAs, 'neurimirs' can be eventually used for potential therapy of blast-induced traumatic brain injury [31].

In summary, our results indicate that acute changes in the expression of genes related to cholinergic and inflammatory pathways in various regions of the brain can play significant roles in the development of chronic neuropathology and neurobehavioral effects after blast exposure. Additionally, modulation of stress and inflammation related microRNAs also play role in blast induced TBI. Targeted therapies focused on cholinergic pathways and microRNA regulation can be feasible approaches to combat against chronic pathophysiological changes after blast exposure.

Conflicts of interest

There is no potential conflict of interest related to this manuscript.

Disclosure

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army, the Navy, or the Department of Defense, USA.

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